SOME ASPECTS OF A SHELL DISEASE IN THE HAWAIIAN FRESHWATER SHRIMP, ATYA BISULCATA (RANDALL)

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INTRODUCTION

A disease syndrome characterized by dark necrotic areas of the exoskeleton has been reported in a number of aquatic crustaceans (Rosen 1970). The syndrome has been called black spot disease, brown spot disease, rust disease, burnt disease, and shell disease. Shell disease was reported in the American lobster, Homarus americanus, by Hess (1937). The disease in this species was thought to be promoted by crowded condition of the lobster holding pens. Similarly, Alaskan king crabs, Paralithodes sp., often developed necrotic "rust" spots when held in captivity. The "rust" appeared to be superficial infections occurring most commonly on the ventral body area at natural breaks and abraded surfaces (Bright, Durham, & Knudsen 1960). Rosen (1967) described a necrotic shell disease of American blue crab, Callinectes sapidus, collected from shedding pens in Crisfield Harbor, Maryland. The crabs had been crowded into enclosures until ecdysis yielded the more desirable "soft-shell" crabs. While the shell disease syndrome is most discernible among crustaceans held in captivity, its occurrence has been reported among crustaceans in their natural habitat. More (1969) reported severely eroded carapaces among blue crabs, C. sapidus, caught in Galveston Bay, Texas. And recently Iversen and Beardsley (1976) reported shell disease among marine crustaceans of South Florida. They found pitted, darkened carapaces among the commercially important stone crab Menippe mercenaria, and several other species.

Shell disease has also been reported in a freshwater prawn, Macrobrachium rosenbergii (Sindermann 1974) and is believed to occur among pond reared specimens in Hawai'i (R. Nakamura, pers. comm.). The presence of shell disease in M. rosenbergii is not surprising since Kubota (1972) reported a severe necrotic shell disease in a closely related species, the Tahitian prawn, M. lar in Kahana Stream on O'ahu.

This study describes the shell disease syndrome in the Hawaiian freshwater atyid shrimp, Atya bisulcata, with emphasis on the frequency of occurrence, the nature of the lesion, and its etiology.

MATERIALS AND METHOD

Collection and Examination of Shrimp

Shrimp specimens were captured from various coastal streams in an area bounded by Hilo to the south and Waikamalo Stream to the north. In this area, characterized by high annual rainfall, streams run throughout the year and support some of the largest populations of atyid shrimps on the island. The majority of our specimens were collected from the Wailuku River, which borders Hilo, and Pukihae Stream a few miles to the north.

In the Wailuku River with its deep pools, shrimps were captured by diving with hand-held scoop nets of 0.25-inch mesh. In smaller, shallow streams, the net was simply held underwater and rocks immediately upstream were overturned. The dislodged shrimps were then swept into the nets and captured. Specimens were transported back to the laboratory in styrofoam chests for examination before returning them to the stream. In some cases, specimens were preserved in 2% formalin for later examination.

Examination of Specimens for Lesions

All specimens brought into the laboratory were initially examined with a Nikon SMZ Stereomicroscope at 10X magnification with an attached ring lamp for illumination. Live specimens had to be cooled in ice water at 5-7°C to facilitate handling of the otherwise very active shrimp. The numbers, size, and location of lesions were noted along with the size and sex of each shrimp. Specimens selected for scanning electron microscopy (SEM) were dissected, dehydrated in an alcohol/acetone series and followed by critical-point drying in amyl acetate or acetone to minimize shrinkage and distortion (Hayat 1978). Specimens were then mounted on aluminum stubs with colloidal silver paint and coated with evaporated gold. Examination was made with an ETEC Autoscan Model U-1 at 20 kv accelerating voltage.

Isolation of Chitinoclastic Bacteria

Lesions selected from newly captured shrimp were aseptically excised and macerated in a sterile mortar and pestle to form a fine slurry. The resultant slurry was diluted 10-fold in sterile water and a 0.1 ml aliquot inoculated onto chitin overlay agar by the spread plate method. Inoculated plates were incubated at 25°C for a minimum of a week and observed for signs of chitin digestion. Chitinoclastic colonies showed a clear halo around the colony as a result of the disolution of the opaque chitin particles (Skerman 1959). Colonies showing distinct chitinoclastic activity were isolated, purified, and maintained on nutrient agar.

Induction of Lesions

To induce lesions, specimens were abraded carefully to remove just the epicuticle. This was accomplished by using a high speed hand drill fitted with a fine abrasive bit. To facilitate handling, the shrimps were first cooled in ice water at 5-7°C for 10 min and strapped onto a polyethylene foam sheet (Nalgene) with nichrome wire hoops. Just prior to abrading, the shell was swabbed with 70% ethanol.

Abraded animals were then rinsed in tap water and transferred to 500 ml of sterile stream water in 1-liter beakers and covered with aluminum foil. Usually, 3 to 5 animals were contained in each beaker. A buffered washed suspension of a 48-hour culture of chitinoclastic bacteria was added to each beaker to achieve an initial cell density of about 10^5 cells per ml. As a control, an identical beaker was used without inoculated bacteria. Also, a control was set up with 10^{-5} M thimerosal added as a bacterial inhibitor.

To determine whether cuticle abrasion was necessary for lesion formation, the wounds of some specimens were sealed immediately after abrasion. These specimens, sealed with Cutex clear nail polish, were subjected to the same incubation conditions as specimens with fresh abrasions.

The test animals were maintained in their containers at ca. 25°C for up to a month with inspection for lesions initiating after one week.

RESULTS AND DISCUSSION

A total of 3423 specimens were collected from various Hilo coast streams. Lesions were found in 582 (17%) of the specimens. Table 1 is representative of lesion incidence found in three streams during March 1976. The Wailuku River site yielded the highest incidence of lesions. Ka'ie'ie Stream, the smallest of the three, had the lowest incidence. Of the three streams, Wailuku River sites consistently yielded the highest incidence of shell lesions. This may be related, in part, to the physical-chemical characteristics of the stream. The greatest volume, water velocity, and silt load are found in the Wailuku River. High silt load and lesion incidence might be related since it has been suggested that abrasive action in the environment could lead to lesion formation (Bright et al. 1960).

The data in Table 1 also shows a significantly higher proportion of female shrimp developing lesions. Not only did female shrimp develop lesions more frequently, they also had more lesions per individual. In this sample period, some females had as many as 30 lesions. Almost all had more than one. By contrast, males often had but a single lesion.

This difference may be explained in part by the observation that most of the females were bearing eggs. During their ovigerous condition, the females do not molt. Hence, lesions have a longer period in which to develop and to become visible. By contrast, the males have no such constraint and molt more frequently. Upon ecdysis, the animal is generally freed of its lesions. In some individuals, however, previous infection is evidenced by a "scar" or a deformation of the new exoskeleton at the site of the former lesion. This deformed cuticle is often the site of subsequent infection.

Extensive sampling of Wailuku River sites revealed a definite seasonal trend in lesion incidence. While lesions occurred among specimens throughout the year, the highest incidences were found in the winter months. A peak of 92% lesion occurrence was reached in February when the water temperature was 14°C. The lowest incidence of 4.7% was recorded in July when water temperature was 24°C. It appeared that the trend in lesion occurrences was inversely related to stream temperature. This unexpected observation may be explained by the fact that lower water temperature would retard the rate of molting, i.e., the intermolt period was lengthened. Again, longer retention of the exoskeleton allows for greater development of the necrotic lesions.

The distribution of lesions on the body surface of A. bisulcata is shown in Table 2. The areas most frequently affected were the cephalothorax and the abdominal segments. This is not surprising since these two body parts offer the greatest exposed surface areas. What is surprising is that the vast majority of lesions occurred on the dorsal or lateral surfaces of the shrimp. Rosen (1967) had shown the ventral surfaces of the blue crab to be the site of intense necrotic lesions. Similarly, Bright et al. (1960) attributed the intensity of lesions occurring on the ventral surfaces of Alaskan king crab to mechanical abrasion by the substrate. It appears that the ventral surfaces and appendages of A. bisulcata are relatively protected from abrasive water-borne silt particles and are, therefore, less susceptible to damage and lesion formation.

Current opinion indicates that exoskeletal lesions in crustacea are in part due to chitin-digesting microorganisms--particularly bacteria. Rosen (1970) and Cook and Lofton (1973) have suggested a causal role for chitinoclastic bacteria. In their studies, the bacteria with chitin-digesting ability had been isolated from diseased hosts. However, controlled reinfection experiments were lacking. In this study, chitinoclastic bacteria were consistently isolated from lesions of A. bisulcata (Table 3). For comparison, lesions of M. lar from the same stream were also examined and found to consistently yield chitinoclastic bacteria. It was found that even the "normal" cuticle of the atyid shrimp would occasionally yield chitinoclastic bacteria. The chitinoclastic bacteria isolated from normal cuticle may have been associated with undetected early stage

lesions. Or they may be part of the normal microflora. Chitinoclastic bacteria are anything but rare in the stream environment. At times they reached over 50,000 per ml of stream water and made up a substantial proportion of all the bacteria present in streams we sampled.

The chitinoclastic bacteria isolated from A. bisulcata were of two types. The majority of isolates were gram negative rods, motile by polar flagella, facultatively anaerobic, and glucose fermentors. These isolates appear to fit the description of the genus Beneckea as proposed by Baumann, Baumann, and Mandel (1971). The other isolate was characterized by bright orange colonies on agar plates and consisted of gram negative, slender rods with gliding motility. The gliding motility is typical of members of the genus Cytophaga. Both the Beneckea and Cytophaga type of isolates showed strong chitinoclastic activity under aerobic condition, but none when incubated anaerobically.

To establish the etiology of the necrotic lesions, pure cultures of chitinoclastic bacteria were used in reinfection experiments. A particularly active Beneckea type, designated WCh-1, was used for the induction of lesions. Table 4 shows that A. bisulcata, abraded to damage the outer cuticle surface, always developed necrotic lesions when confined in a system with abundant chitinoclastic bacteria. Likewise, abraded shrimp confined in raw stream water also consistently developed lesions. Water as taken directly from the stream always contained numerous chitinoclastic bacteria. It was believed that these autochthonous bacteria serve as an infectious reservoir in the stream. When stream water was sterilized (Millipore membrane filter, 0.45 μm pore) or thimerosal added as a bacteriocidal agent, necrotic lesion formation was markedly suppressed. In the few instances where lesions did form in supposedly "sterile" conditions, chitinoclastic bacteria were subsequently found. It appears that residual fecal pellets were a source of the bacteria which contaminated the system and overwhelmed the suppressive capability of the thimerosal.

To show that epicuticular damage was indeed necessary for the initiation of lesions, as suggested by Rosen (1970), abraded test animals were compared to similarly treated specimens in which the abrasions were sealed water tight with clear nail polish. The results in Table 5 show a striking difference. Damaged cuticle when exposed to raw stream water containing chitinoclastic bacteria invariably developed necrotic lesions. Those with sealed wounds never developed lesions unless the seals were faulty and leaking.

In all attempted isolations, the artificially induced lesions yielded chitinoclastic bacteria--almost exclusively of the Beneckea type. Scanning electron microscopy of the naturally occurring lesion and artificially induced lesion showed a remarkable similarity. In both cases, extensive erosion of the cuticle is evident and large aggregations of bacteria are seen. By contrast, areas free of abrasive damage or erosion are essentially

free of bacteria. The activity of these bacteria on the chitinous substrate very closely resembles that described by Akin and Amos (1975) for cellulolytic bacteria and their degradation of cellulosic plant material. In both cases, the production of exoenzymes is likely responsible for hydrolytic degradation of the substrate.

This study has shown that necrotic exoskeletal lesions are common to the endemic Hawaiian freshwater shrimp, A. bisulcata. The causal agent of these lesions have been shown to be chitinoclastic bacteria which are ubiquitous in the stream environment and probably a component of the shrimp's microflora. Stream conditions which lead to damage of the cuticle--primarily the epicuticle layer--exposed the cuticle to bacterial invasion. Establishment of chitinoclastic bacteria on the cuticle and subsequent degradation of the chitinous substrate result in formation of a necrotic lesion. The dark coloration characteristic of these necrotic areas is due to melanization of hemocytes which aggregate underneath the developing lesion.

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TABLE 1. Occurrence of exoskeletal lesions in Atya bisulcata from selected Hawai'i Island streams (% in parentheses).

Sampling Site	Number of Specimens			Specimens with Lesions		
	Total	Female	Male	Total	Female	Male
Wailuku River	242	30	212	163 (67.4)	27 (90.0)	136 (64.2)
Ka'ie'ie Stream	243	45	198	17 (7.0)	9 (20.0)	8 (4.0)
Pukihae Stream	363	146	216	135 (37.3)	64 (43.8)	71 (32.9)

TABLE 2. The location of exoskeletal lesions in Atya bisulcata from Wailuku River, Hawai'i.

Body Part	Number* of Lesions	Percent of Total
Cephalothorax	27	33.8
Abdomen Segments	47	58.8
Telson & Uropod	2	2.5
Walking Legs	2	2.5
Swimmerets	2	2.5
Total	80	

* 36 lesions on left side, 30 lesions on right side, and 14 lesions located medially

TABLE 3. Isolation of chitinoclastic bacteria from exoskeletal lesions.

Source	Number of Lesions Sampled	Lesions Yielding Chitinoclasts	
		Number	Percent
Atya bisulcata lesion	24	23	95.8
Macrobrachium lar lesion	17	14	82.3
A. bisulcata normal cuticle	24	4	16.7

TABLE 4. Induced exoskeletal lesions in *Atya bisulcata*.

Test Conditions	Total Specimens	Percent	
		With Lesion	Without Lesion
Chitinoclastic Bacteria in sterile stream water	33	100	0
Raw stream water	16	94	6
Sterile stream water with Thimerosal, 10^{-5} M	20	15	85

TABLE 5. The development of exoskeletal lesions in *Atya bisulcata* with abrasions. (Specimens were held in raw stream water at 25°C for a minimum of 8 days.)

Treatment	Total Number of Specimen	Number of Specimens	
		Developing Lesions	Without Lesions
Abraded only	25	25	0
Abraded and sealed	15	1	14